MOLECULAR EVOLUTION OF GPCRS

26RFa/GPR103

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Abstract

Neuropeptides possessing the Arg-Phe-NH₂ (RFamide) motif at their C-termini (designated as RFamide peptides) have been characterized in a variety of animals. Among these, neuropeptide 26RFa (also termed QRFP) is the latest member of the RFamide peptide family to be discovered in the hypothalamus of vertebrates. The neuropeptide 26RFa/QRFP is a 26-amino acid residue peptide that was originally identified in the frog brain. It has been shown to exert orexigenic activity in mammals and to be a ligand for the previously identified orphan G protein-coupled receptor, GPR103 (QRFPR). The cDNAs encoding 26RFa/QRFP and QRFPR have now been characterized in representative species of mammals, birds, and fish. Functional studies have shown that, in mammals, the 26RFa/QRFP–QRFPR system may regulate various functions, including food intake, energy homeostasis, bone formation, pituitary hormone secretion, steroidogenesis, nociceptive transmission, and blood pressure. Several biological actions have also been reported in birds and fish. This review summarizes the current state of identification, localization, and understanding of the functions of 26RFa/QRFP and its cognate receptor, QRFPR, in vertebrates.

Key Words

- ▶ 26RFa/QRFP
- ▶ food intake
- ▶ G protein-coupled receptor
- ▶ hypothalamus
- ▶ neuropeptide

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Introduction

Neuropeptides that possess the Arg-Phe-NH₂ motif at their C-termini (i.e., RFamide peptides) have been characterized both in invertebrates and vertebrates. The first RFamide peptide to be identified was the cardioexcitatory peptide Phe-Met-Arg-Phe-NH₂ (FMRamide), which was isolated from the ganglia of the Venus clam *Macrocallista nimbosa* (Price & Greenberg 1977). Since then, a number of RFamide peptides have been identified in invertebrates, where these peptides seem to act as neurotransmitters and neuromodulators (for review, see Walker *et al.* (2009)).

A number of immunohistochemical studies that used antisera against FMRamide suggested that the nervous system of vertebrates also contained neuropeptides immunologically related to FMRamide (Raffa 1988, Vallarino *et al.* 1991, 1994, 1995, Rastogi *et al.* 2001). In fact, several neuropeptides harboring the RFamide sequence at their C-terminal end have been characterized in the brain of various vertebrates. In the past, the existence of five groups within the RFamide peptide family has been recognized in vertebrates, namely

the neuropeptide FF (NPFF) group, the prolactin-releasing peptide (PrRP) group, the gonadotropin-inhibitory hormone (GnIH) group, the kisspeptin group, and the 26RFa/QRFP group (for reviews, see Ukena & Tsutsui (2005), Bruzzone *et al.* (2006), Osugi *et al.* (2006), Tsutsui & Ukena (2006), Tsutsui (2009), Tsutsui *et al.* (2010a,b), Chartrel *et al.* (2011), Leprince *et al.* (2013); Fig. 1). These RFamide peptides have been shown to exert important neuroendocrine, behavioral, sensory, and autonomic functions (for reviews, see Chartrel *et al.* 2002, 2006a, Ukena & Tsutsui 2005, Tsutsui & Ukena 2006). Among these vertebrate RFamide peptides, NPFF is well documented as a morphine modulatory peptide (Panula *et al.* 1999). In addition, GnIH and kisspeptin appear to play key roles in the regulation of the reproductive axis (Tsutsui *et al.* 2010b). This review summarizes the current state of knowledge on the molecular evolution and functions of 26RFa/QRFP, the latest member of the RFamide peptide family to be discovered in vertebrates, and of its cognate receptor, QRFPR. This review also indicates future directions in this research field.

Unity and diversity of the structure of 26RFa/QRFP in vertebrates

The 26-amino acid residue RFamide peptide, 26RFa/QRFP, was identified for the first time in the brain of an amphibian species (Chartrel *et al.* 2003). An antibody against the RFamide motif was used to screen, by RIA, peptide fractions purified from a brain extract of the European green frog (*Rana esculenta*). After HPLC purification, the sequence of the isolated substance was analyzed by mass spectrometry MS/MS fragmentation; it turned out to be a 26-amino acid peptide possessing the RFamide motif at its C-terminus, namely VGTALGSLAEELNGYNRKKGGFSRFamide. This neuropeptide had not been reported in any animals previously and was designated as 26RFa (Fig. 2A; Chartrel *et al.* 2003).

The amino acid sequence of frog 26RFa was employed to identify the cDNA encoding the counterpart of 26RFa in rat and humans (Chartrel *et al.* 2003). Concurrently, two other research groups independently identified 26RFa/QRFP precursors using a bioinformatic approach in the rat, mouse, bovine, and human genomes and paired 26RFa/QRFP with a previously identified orphan G protein-coupled receptor (GPCR), GPR103, also known as AQ27 or SP9155 (Fukusumi *et al.* 2003, Jiang *et al.* 2003; Fig. 2B). *GPR103* has thus been renamed *QRFPR* by the HUGO Gene Nomenclature Committee (<http://www.genenames.org/>). The mature 43-amino acid residue

RFamide peptide was identified from the culture medium of CHO cells that expressed the human peptide precursor (Fukusumi *et al.* 2003). As the N-terminal amino acid was pyroglutamic acid, this RFamide peptide was also named pyroglutamylated RFamide peptide (QRF; Fukusumi *et al.* 2003). Subsequently, the cDNAs encoding the 26RFa/QRFP precursors have been characterized in goldfish (Liu *et al.* 2009), quail (Ukena *et al.* 2010), chicken (Ukena *et al.* 2010), and zebra finch (Tobari *et al.* 2011) (Fig. 2B). Although the *26RFa/qrfp* cDNA has not been characterized in the European green frog, the corresponding sequence in the African clawed frog (*Xenopus tropicalis*) is present in the database (Fig. 2B). Furthermore, homologous sequences have been listed in the genome database of reptilian (lizard) and fish (stickleback, medaka, fugu, and zebrafish) species (Liu *et al.* 2009). These data have revealed the existence of the 26RFa/QRFP-encoding gene in representative species of the whole vertebrate phyla, including fish, amphibians, reptilians, birds, and mammals (Chartrel *et al.* 2011, Ukena *et al.* 2011).

As there are several monobasic processing sites in the 26RFa/QRFP precursor protein, alternative cleavage may yield various N-terminally elongated forms of 26RFa/QRFP (Chartrel *et al.* 2006b, 2011). HPLC analysis combined with RIAs indicated the existence of both 26- and 43-amino acid residue RFamide peptide-like immunoreactivities in the hypothalamus and spinal cord of humans (Bruzzone *et al.* 2006). Indeed, an N-terminally extended peptide of 43 residues, called 43RFa or QRF, has been characterized in rat brain extracts, as well as in PC12 cells and the culture medium of CHO cells that express the human precursor, as described above (Fukusumi *et al.* 2003, Bruzzone *et al.* 2006, Takayasu *et al.* 2006; Fig. 2A). The human and *Xenopus* 26RFa/QRFP precursors may also generate a nine-amino acid peptide, termed 9RFa, located upstream of 26RFa/QRFP (Fig. 2B). However, 9RFa has not been detected in tissue extracts to date. Structure–activity relationship studies have revealed that the synthetic C-terminal heptapeptide (26RFa_{20–26}; GGFSRFamide) is responsible for the biological activity of 26RFa/QRFP (Le Marec *et al.* 2011, Neveu *et al.* 2012). A reverse pharmacological study has demonstrated that 26RFa/QRFP is a natural ligand for the previously identified orphan receptor, GPR103 (QRFPR), as described below (Fukusumi *et al.* 2003, Jiang *et al.* 2003, Takayasu *et al.* 2006).

As reported above, the mature forms of 26RFa/QRFP have been identified in the brains of amphibians and mammals (Chartrel *et al.* 2003, Bruzzone *et al.* 2006, Takayasu *et al.* 2006), but, until recently, the existence of 26RFa/QRFP has not been investigated in birds.

Figure 1

Phylogenetic tree of the RFamide peptide family in vertebrates. Studies over the past decade have demonstrated that the brain of vertebrates produces a variety of RFamide peptides. To date, five groups have been

identified within this family: the neuropeptide FF (NPFF) group, the prolactin-releasing peptide (PrRP) group, the gonadotropin-inhibitory hormone (GnIH) group, the kisspeptin group, and the 26RFa/QRFP group.

Among the RFamide peptide family, only the GnIH group had been found in avian at the time we started this study (for reviews, see Ukena & Tsutsui 2005, Tsutsui & Ukena 2006, Tsutsui 2009, Tsutsui *et al.* 2010a,b). We therefore looked for 26RFa/QRFP in the avian brain and found the presence of a gene encoding the 26RFa/QRFP precursor in chicken after searching the genomic database.

Subsequently, the cDNA of the 26RFa/QRFP precursor was sequenced in the quail hypothalamus (Ukena *et al.* 2010). The quail precursor protein demonstrates 88% overall similarity with the chicken sequence, 47% with the corresponding human sequence, and 40% with the rat sequence (Ukena *et al.* 2011). A Lys-Arg dibasic cleavage site is present in the C-terminal region of the quail and

Figure 2

Alignments of the amino acid sequences of identified 26Rfa/QRFP peptides (A) and their precursor proteins (B) deduced from mammalian (human, bovine, rat, and mouse), avian (chicken, quail, and zebra finch), amphibian (*Xenopus*), and fish (goldfish) cDNAs. The predicted signal peptide sequences are underlined with a dashed line. <E represents pyroglutamic acid. The positions of identified mature peptides in the precursor proteins are underlined with solid lines. The human and *Xenopus* 26Rfa/QRFP precursors may also generate a nine-amino acid peptide, termed 9Rfa (boxed). Fully conserved amino acids are highlighted with red boxes and highly conserved amino acids with gray boxes respectively. The Lys (K)-Arg

(R) dibasic processing sites in birds and *Xenopus*, the single Arg (R) putative processing sites in mammals and fish, and the Gly (G) C-terminal amidation signals are shown in bold. Gaps marked by hyphens were inserted to optimize homology. The GenBank accession numbers of these sequences are as follows: human 26Rfa/QRFP, NP_937823; bovine 26Rfa/QRFP, NP_937865; rat 26Rfa/QRFP, NP_937843; mouse 26Rfa/QRFP, NP_906269; chicken 26Rfa/QRFP, XP_001235089; quail 26Rfa/QRFP, BAI81890; zebra finch 26Rfa/QRFP, BAK32798; *Xenopus tropicalis* 26Rfa/QRFP, XP_002936227; and goldfish 26Rfa/QRFP, ACI46681.

chicken precursor sequences, but not in that of mammalian sequences (Fig. 2B). This indicates that the mature peptide consists of 27 amino acid residues in quail and chicken, unlike the 26 residues in the amphibian 26Rfa/QRFP sequence (Chartrel *et al.* 2003). In fact, MS analysis combined with immunoaffinity purification has revealed that the 27-amino acid sequence corresponds to the mature form of the peptide in the quail hypothalamus,

indicating that the peptide is actually produced from the precursor in the hypothalamus (Ukena *et al.* 2010; Fig. 2A). More recently, a 26Rfa/QRFP ortholog, consisting of 25 amino acids, and the related cDNA have been characterized in the brain of zebra finch (Tobari *et al.* 2011; Fig. 2).

Syntenic analysis of the 26Rfa/QRFP gene revealed that the chromosomal region encompassing the 26Rfa/QRFP gene is highly conserved from amphibians to

human. Indeed, all these regions contain paralogs of several other genes and thus clearly constitute a paralogon (Fig. 3). However, this paralogon has not been preserved in fish (Fig. 3), possibly because of the specific genome duplication and rearrangements that have occurred during the evolution in the fish lineage. To date, the existence of *26RFa/qrfp* gene in coelacanth and lamprey is still unclear (Fig. 3).

Comparative aspects of biological actions of 26RFa/QRFP in vertebrates

Mammals

The mRNAs encoding 26RFa/QRFP and its cognate receptor QRFP are highly expressed in the dorsolateral and mediobasal hypothalamic areas of rodents (Chartrel *et al.* 2003, Takayasu *et al.* 2006, Bruzzone *et al.* 2007).

These two areas are known to be involved in the regulation of energy homeostasis. In a similar way, in human, 26RFa/QRFP-producing cells are localized in the paraventricular and ventromedial nuclei of the hypothalamus (Bruzzone *et al.* 2006), which are also known to regulate food intake. Indeed, i.c.v. injection of 26RFa/QRFP has been demonstrated to stimulate food intake in rodents (Chartrel *et al.* 2003, Do Régo *et al.* 2006, Moriya *et al.* 2006, Takayasu *et al.* 2006, Primeaux *et al.* 2008, 2013, Lectez *et al.* 2009, Primeaux 2011).

In addition to its orexigenic effects, 26RFa/QRFP has been reported to exert a wide range of biological actions (Fig. 4). In an earlier report, i.v. administration of 26RFa/QRFP was found to increase plasma aldosterone levels in a dose-dependent manner in rats (Fukusumi *et al.* 2003). Recently, it has been reported that 26RFa/QRFP and QRFP are present in the human and rat adrenal gland and that 26RFa/QRFP stimulates corticosteroid secretion

Figure 3

Synteny analysis around *26RFa/QRFP* gene loci. Orthologous or paralogous genes are linked by horizontal lines. The *26RFa/QRFP* genes are shown white in black boxes. The nucleotide position of each gene on the chromosome is shown under each gene. The GenBank accession numbers

of *26RFa/QRFP* genes are as follows: human *26RFa/QRFP*, AB109625.1; mouse *26RFa/QRFP*, AB109628.1; chicken *26RFa/QRFP*, XM_001235088.2; *Xenopus tropicalis* *26RFa/qrfp*, XM_004916673.1; zebrafish *26RFa/qrfp*, XP_00133883; medaka *26RFa/qrfp*, XP_004073955.1.

Figure 4

Demonstrated biological actions of 26RFa/QRFP-QRFP system in vertebrates. Both 26RFa/QRFP and QRFP have been found to exert a wide array of biological activities.

by human adrenocortical cells (Ramanjaneya *et al.* 2013). In the rat pancreas, glucose-evoked insulin secretion is reduced by perfusion of 26RFa/QRFP (Egido *et al.* 2007). In the adipocyte cell line 3T3-L1, 26RFa/QRFP inhibits isoproterenol-induced lipolysis (Malumba *et al.* 2010). As 3T3-L1 cells express the QRFP-encoding gene, it appears that 26RFa/QRFP may act in an autocrine/paracrine manner to regulate adipogenesis (Malumba *et al.* 2010). According to Alonzeau *et al.* (2013), 26RFa/QRFP is also expressed in human prostate cancer and stimulates the neuroendocrine differentiation and migration of cancer cells. Administration of 26RFa/QRFP in the brain increases plasma luteinizing hormone (LH) levels in both sexes in rat (Navarro *et al.* 2006, Patel *et al.* 2008) and stimulates prolactin and growth hormone secretion in male rhesus monkeys (Qaiser *et al.* 2012, Wahab *et al.* 2012). Intrathecally administered 26RFa/QRFP induces analgesic effects in rat under formalin and carrageenan tests (Yamamoto *et al.* 2008). Central injection of 26RFa/QRFP in mice causes a rise in blood pressure and heart rate (Takayasu *et al.* 2006). Mice deficient in the receptor for 26RFa/QRFP (*Qrfpr*) suffer from osteopenia (Baribault *et al.* 2006). This observation indicates that 26RFa/QRFP plays a major role in bone formation, via QRFP that is expressed in bone (Baribault *et al.* 2006).

Non-mammalian vertebrates

In goldfish, quantitative RT-PCR analysis demonstrated high expression of *26RFa/qrfp* mRNA in the hypothalamus, optic tectum-thalamus, and testis. The expression of

26RFa/qrfp mRNA in the hypothalamus is augmented at 4 days after food deprivation (Liu *et al.* 2009). In addition, serum LH levels are significantly increased at 1 h, but not at 3 and 6 h after i.p. injection of 26RFa/QRFP (Liu *et al.* 2009). As 26RFa/QRFP has no effect on LH release from pituitary cells in primary culture, it is thought that, in fish, the peptide may stimulate the gonadotropic axis by acting exclusively at the hypothalamic level. These results suggest that 26RFa/QRFP regulates energy homeostasis and the hypothalamic-pituitary-gonadal axis in fish, as also observed in mammals.

In birds, the expression of *26RFa/QRFP* mRNA in the quail brain has been investigated in different brain regions, i.e., the cerebrum, diencephalon, mesencephalon, and cerebellum, by quantitative PCR analysis. A high level of expression of *26RFa/QRFP* mRNA is present in the diencephalon, including the hypothalamus, while *26RFa/QRFP* mRNA is almost undetectable in other brain regions (Ukena *et al.* 2010). In colchicine-treated birds (quail and chicken), 26RFa/QRFP-immunoreactive cell bodies were found only in the anterior hypothalamic nucleus in the diencephalon (Ukena *et al.* 2010). Furthermore, *in situ* hybridization has shown specific expression of *26RFa/QRFP* mRNA in the anterior hypothalamic nucleus in the chick brain, and the distribution of *26RFa/QRFP* mRNA-containing perikarya clearly matches with that of 26RFa/QRFP-immunoreactive neurons (Ukena *et al.* 2010). In the zebra finch, *in situ* hybridization analysis has revealed that expression of *26RFa/QRFP* mRNA is localized to the anterior-medial hypothalamic area, the ventromedial nucleus of the hypothalamus, and the lateral hypothalamic area (Tobari *et al.* 2011). These neuroanatomical data suggest that, in birds, 26RFa/QRFP produced in the hypothalamus participates in the control of feeding behavior, as shown previously in rodents (Chartrel *et al.* 2006b, Do Régio *et al.* 2006, Moriya *et al.* 2006, Primeaux *et al.* 2008).

To assess the above speculation, the effect of central injection of 26RFa/QRFP has been surveyed in both broiler and layer chick lines. I.c.v. injection of 26RFa/QRFP stimulates feeding behavior in broiler chicks, but not in layer chicks (Ukena *et al.* 2010). It is likely that the different effects in these two chick lines can be explained by the following reports. It has been demonstrated that the effect of 26RFa/QRFP on feeding behavior in rodents differs according to the energy status and/or the species (Primeaux *et al.* 2013). Although 26RFa/QRFP hardly affects food intake in normally fed rats (Fukusumi *et al.* 2003, Kampe *et al.* 2006, Patel *et al.* 2008), at least under a low-fat diet (Primeaux *et al.* 2008), 26RFa/QRFP induces a

marked orexigenic effect in mice and food-restricted rats (Chartrel *et al.* 2003, 2005, Do Régo *et al.* 2006, Moriya *et al.* 2006, Takayasu *et al.* 2006, Lectez *et al.* 2009). In addition, it has been demonstrated that 26RFa/QRFP selectively increases the intake of a high-fat diet in rats (Primeaux *et al.* 2008, 2013, Primeaux 2011). On the other hand, to determine the biologically active core of 26RFa/QRFP, the effect of a synthetic C-terminal octapeptide (26RFa-8; KGGFAFRFamide) of 26RFa/QRFP has been tested on feeding behavior of chicken. This C-terminal sequence is highly conserved from fish to mammals (Fig. 2). The synthetic C-terminal octapeptide, 26RFa-8, stimulates food intake in broiler chicks, but not in layer chicks, in much the same manner as the full-length peptide (Ukena *et al.* 2010). Consistent with this observation, a synthetic C-terminal heptapeptide of 26RFa/QRFP (26RFa_{20–26}; GGFSFRFamide) exerts an orexigenic effect in mice (Do Régo *et al.* 2006). In addition, 26RFa_{20–26} evokes a significant increase in serum LH levels in female rats (Navarro *et al.* 2006). Taken together, it appears that the C-terminal region of 26RFa/QRFP is responsible for the biological activity of the peptide. In addition to the chick data, it has been reported that central injection of 26RFa/QRFP in free-feeding male zebra finches stimulates food intake for 24 h, without a change in body mass (Tobari *et al.* 2011). These results also indicate that 26RFa/QRFP exerts an orexigenic activity in various avian species.

Comparative aspects of QRFPR in vertebrates

Mammals

In humans, 26RFa/QRFP has been found to be an endogenous ligand for the orphan receptor, GPR103 (QRFPR), which is a class A GPCR (Fukusumi *et al.* 2003, Jiang *et al.* 2003). QRFPR shares relatively high sequence similarity with other RFamide receptors, notably those for NPFF, PrRP, kisspeptin, and GnIH, and to a lesser extent with the other peptidergic receptors for neuropeptide Y (NPY), galanin, orexin, and cholecystokinin (Lee *et al.* 2001, Jiang *et al.* 2003). Surprisingly, 26RFa/QRFP displays a moderate affinity for NPFF2 (NPFFR2, the receptor for NPFF) and a low affinity for NPFF1 (NPFFR1, the receptor for GnIH) (Gouardères *et al.* 2007). In addition, QRFPR possesses several characteristic features of class A GPCRs, such as i) a disulfide bridge between the two Cys (C) residues located in the first and second extracellular loops (EL1 and EL2), ii) the existence of an Asp (D) residue within the second transmembrane domain (TM2) that

seems to play a pivotal role in G protein coupling, iii) a conserved Glu (E)-Arg (R) doublet sequence at the N-terminal end of the second intracellular loop (IL2), and iv) three conserved residues, i.e., Phe (F), Pro (P) and Asn (N), within TM6 and TM7, which are crucial for receptor activation (Fig. 5). QRFPRs with 26 and 43 amino acid residues bind to QRFPR with high affinity (EC_{50} =3.2 and 0.52 nM respectively) (Fukusumi *et al.* 2003, Jiang *et al.* 2003). It has also been demonstrated that 26- and 43-amino acid residue QRFPRs inhibit cAMP formation with similar efficacy in QRFPR-transfected CHO cells (Fukusumi *et al.* 2003). Furthermore, 26RFa/QRFP markedly increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in a pertussis toxin-independent manner. These results suggest that QRFPR is coupled to a $G_{i/o}$ and/or to a G_q protein (Fukusumi *et al.* 2003). The affinity and potency of the C-terminal heptapeptide 26RFa_{20–26} (GGFSFRFamide) have been investigated and were found to be lower than those of 26RFa/QRFP. These data indicate that this heptapeptide is a relatively weak ligand for QRFPR (Fukusumi *et al.* 2003, Le Marec *et al.* 2011). Furthermore, it has been reported that 26RFa/QRFP enhances corticosteroid secretion in human adrenocortical cells by regulating key steroidogenic enzymes involving MAPK/PKC and Ca^{2+} signaling pathways via QRFPR (Ramanjaneya *et al.* 2013).

In contrast to humans, who only have a single QRFPR-encoding gene, two isoforms of the receptor for 26RFa/QRFP have been characterized in rodents. These 26RFa/QRFP receptor isoforms have been designated as QRFPR1 and QRFPR2 in rat and mouse (Kampe *et al.* 2006, Takayasu *et al.* 2006); 26RFa/QRFP stimulates inositol trisphosphate in rat QRFPR1 and QRFPR2 with similar efficacy (Kampe *et al.* 2006) and binds to mouse QRFPR1 and QRFPR2 with similar affinity (Takayasu *et al.* 2006).

The distribution of QRFPR mRNA and its peptide binding sites have been studied by *in situ* hybridization and autoradiography respectively. In rat, *Qrfpr* mRNA-containing cells are notably expressed in the midbrain, the pons, and the medulla oblongata, while 26RFa/QRFP-binding sites are widely distributed throughout the brain and spinal cord (Bruzzzone *et al.* 2007). These results suggest that 26RFa/QRFP can bind to a receptor(s) other than QRFPR. Indeed, it has been found by competition experiments that 26RFa/QRFP interacts with NPFF2, the cognate receptor for NPFF (Bruzzzone *et al.* 2007). The widespread distribution of 26RFa/QRFP-binding sites suggests that 26RFa/QRFP exerts multiple functions in the brain and spinal cord that are mediated

Figure 5

Alignment of the amino acid sequences of the G protein-coupled receptor for 26RFa/QRFP, QRFP, in mammals (human and rat), birds (chicken and zebra finch), frog (*Xenopus*), and fish (zebrafish). Fully conserved amino acids are highlighted with green boxes and highly conserved amino acids with gray boxes. Putative transmembrane domains (TMD) are underlined. The disulfide bridge between the two Cys (C) residues located in the first and second extracellular loops is indicated by a line. The Asp (D) residue in TMD2 involved in G protein coupling, the conserved Glu (E)-Arg (R)

residues in the second intracellular loop, and the conserved Phe (F), Pro (P), and Asn (N) residues in TMD6 and TMD7 are represented by colored letters. A hyphen has been inserted to obtain optimal homology. The GenBank accession numbers of these sequences are as follows: human QRFP, NP_937822; rat QRFP, NP_937842; chicken QRFP, NP_001120642; zebra finch QRFP, NP_001243137; *Xenopus tropicalis* QRFP, NP_001072295; and zebrafish Qrfp, XP_001920042.

Figure 6

Synteny analysis around *QRFPR* gene loci. Orthologous or paralogous genes are linked by horizontal lines. The *QRFPR* genes are shown white in black boxes. The nucleotide position of each gene on the chromosome is shown under each gene. The GenBank accession numbers of *QRFPR* genes are as follows: human *QRFPR*, JF810892.1; mouse *Qrfpr1*, BC096610.1; chicken *QRFPR*, NM_001127170.1; *Xenopus tropicalis* *qrfrp*, NM_001078827.1; and medaka *qrfrp*, XP_004080459.1. Ensembl genome

database accession numbers are as follows: mouse *Qrfpr2*, ENSMUSG00000029917; zebrafish *qrfrp1*, ENSDARG00000039349; zebrafish *qrfrp2*, ENSDARG00000068422; zebrafish *qrfrp3*, ENSDARG00000092652; coelacanth *qrfrp*, ENSLACG00000016226; and sea lamprey *qrfrp*, ENSPMAG00000005451. GENSCAN (<http://genes.mit.edu/GENSCAN.html>) was used to predict putative coelacanth *Qrfpr3* precursor protein.

by at least two distinct receptors, *QRFPR* and *NPFF2* (Bruzzone *et al.* 2007).

Non-mammalian vertebrates

In birds, the cDNAs encoding *QRFPR* have been characterized in the brain of chicken and zebra finch (Ukena *et al.* 2010, Tobari *et al.* 2011). The sequence of chicken *QRFPR* is highly similar to those of human and rat *QRFPR* (Fig. 5). The action of 26RFa/QRF on chicken *QRFPR* has been studied by measuring $[Ca^{2+}]_i$ in HEK293T cells that had been transiently transfected with chicken *QRFPR*. In these cells, 26RFa/QRF increases $[Ca^{2+}]_i$ in a dose-dependent manner, with an EC_{50} value of around 40 nM (Ukena *et al.* 2010). The mRNA of *QRFPR* is widely expressed in chicken and zebra finch brains and the highest concentration of mRNA is observed in the diencephalon (Ukena *et al.* 2010, Tobari *et al.* 2011). As the mRNA of *QRFPR* is expressed in the brain outside the diencephalon in chicken, as it is in rat (Bruzzone *et al.* 2007), 26RFa/QRF may exert multiple functions in addition to regulating food intake (Ukena *et al.* 2010).

Synteny analysis has revealed the existence of species-specific paralogous genes of *QRFPR* in mouse, zebrafish and coelacanth (Fig. 6). These paralogous genes may have emerged along with the species-specific gene or genome duplications that occurred during the course of vertebrate

evolution. Phylogenetic analysis data are consistent with synteny analysis (Fig. 7). Although there are homologous sequences to *QRFPR* in the genome database of *Xenopus*, zebrafish, coelacanth and lamprey (Figs 5 and 6), *Qrfpr* has been studied only in mammals and birds. Further characterization of *QRFPR* is thus needed to determine the functional significance of the 26RFa/QRF–*QRFPR* system in other vertebrate phyla, such as reptilians, amphibians, and fish.

Conclusions and future directions

The neuropeptide 26RFa/QRF belongs to the most recently identified group of the RFamide peptide family and was first identified in the brain of the European green frog. Subsequently, the cDNAs encoding the 26RFa/QRF precursors have been characterized in various animals, including goldfish, quail, chicken, zebra finch, mouse, rat, bovine, and humans, and these analyses have shown the existence of the 26RFa/QRF-encoding gene in representative species of the vertebrate phylum. In mammals, 26RFa/QRF has been found to be a high-affinity endogenous ligand for the previously identified orphan GPCR, GPR103 (*QRFPR*). In rodents and monkeys, 26RFa/QRF exerts diverse biological actions, including regulation of food intake and energy homeostasis, hormone secretion, nociception, and bone formation.

Figure 7

Phylogenetic analysis of QRFPR precursor proteins. *Drosophila melanogaster* peptide GPCR was used as an outgroup. NPY receptors are included in the phylogenetic tree as a reference group of vertebrate GPCR. Scale bar refers to a phylogenetic distance of 0.1 nucleotide substitutions per site. Numbers on the branches indicate bootstrap percentage following 1000

replications in constructing the tree. The GenBank accession numbers of the *NPY1R* genes are as follows: human *NPY1R*, NM_000909; mouse *Npy1r*, NM_010934; chicken *NPY1R*, NM_001031535; anole lizard *NPY1R*, XM_003221700; *Xenopus laevis* *npy1r*, NM_001085879; zebrafish *npy1r*, NM_001102391; and *Drosophila melanogaster* peptide GPCR, AY217746.1.

Recently, the mature sequences of 26RFa/QRFPR have been identified by structural analysis in quail and zebra finch. In birds, as in mammals, 26RFa/QRFPR-producing neurons are only located in the hypothalamus, while QRFPR is widely distributed throughout the brain. In birds, 26RFa/QRFPR also exerts an orexigenic action, as it does in rodents, and a

similar effect of 26RFa/QRFPR has been suggested in fish, because of upregulation of *26RFa/qrfp* mRNA by a negative energy state. Thus, the structure, distribution pattern, and biological actions of the 26RFa/QRFPR–QRFPR system have been conserved across the vertebrate phylum, from fish to mammals. However, further studies are clearly required to

fully elucidate the molecular evolution and functional significance of the 26RFa/QRFP–QRFPR pair in vertebrates. In particular, *in vitro* and *in vivo* studies on development, morphogenesis, and behavior in non-mammalian model organisms, such as *Xenopus* and zebrafish, should bring to light previously unknown physiological actions of the 26RFa/QRFP–QRFPR system. Recent studies have shown that a number of neuropeptide/GPCR pairs initially discovered in vertebrates/deuterostomes actually possess homologs in protostomes (Sherwood *et al.* 2006, Roch *et al.* 2011, Frooninckx *et al.* 2012, Grimmelikhuijzen & Hauser 2012, Mirabeau & Joly 2013). It would thus be interesting to look for the existence of 26RFa/QRFP and/or QRFPR orthologs in representative species of protostomes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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